

Dartmouth College

Dartmouth Digital Commons

Open Dartmouth: Peer-reviewed articles by
Dartmouth faculty

Faculty Work

3-2-1990

N-Ethylmaleimide-Sensitive Protein(s) Involved in Cortical Exocytosis in the Sea-Urchin Egg - Localization to Both Cortical Vesicles and Plasma-Membrane

Robert C. Jackson
Dartmouth College

Paul A. Modern
Dartmouth College

Follow this and additional works at: <https://digitalcommons.dartmouth.edu/facoa>



Part of the [Cell Biology Commons](#)

Dartmouth Digital Commons Citation

Jackson, Robert C. and Modern, Paul A., "N-Ethylmaleimide-Sensitive Protein(s) Involved in Cortical Exocytosis in the Sea-Urchin Egg - Localization to Both Cortical Vesicles and Plasma-Membrane" (1990). *Open Dartmouth: Peer-reviewed articles by Dartmouth faculty*. 1750.
<https://digitalcommons.dartmouth.edu/facoa/1750>

This Article is brought to you for free and open access by the Faculty Work at Dartmouth Digital Commons. It has been accepted for inclusion in Open Dartmouth: Peer-reviewed articles by Dartmouth faculty by an authorized administrator of Dartmouth Digital Commons. For more information, please contact dartmouthdigitalcommons@groups.dartmouth.edu.

***N*-ethylmaleimide-sensitive protein(s) involved in cortical exocytosis in the sea urchin egg: localization to both cortical vesicles and plasma membrane**

ROBERT C. JACKSON* and PAUL A. MODERN

Department of Biochemistry, Dartmouth Medical School, Hanover NH 03756, USA

* Author for correspondence

Summary

The exocytotic release of secretory products from fragments of sea urchin egg cortex has been shown to be inhibited by covalent modification of membrane sulfhydryl groups with *N*-ethylmaleimide (NEM). Exocytotically competent preparations of reconstituted cortex, formed by recombination of purified cortical vesicles (CVs) with fragments of egg plasma membrane (PM) were also inhibited by treatment with NEM. The cellular localization of sulfhydryl-containing constituent(s) responsible for inhibition was investigated by treating CVs and/or PM with NEM prior to reconstitution. Both native cortex and cortex reconstituted with NEM-treated components were challenged with calcium-containing buffers. Exocytosis was monitored by phase-contrast microscopy, and quantitated by light scattering. Evidence for CV-PM fusion was obtained with an immunofluorescence-based assay that permits

visualization of the transport of CV content proteins across the PM. Cortex reconstituted by recombination of NEM-treated CVs with untreated PM or by recombination of untreated CVs with NEM-treated PM was exocytotically competent, whereas cortex formed by recombination of NEM-treated CVs with NEM-treated PM was inactive. These results: (1) support the hypothesis that the mechanism of exocytosis in native and reconstituted cortex is the same; (2) provide evidence that both CV and plasma membranes participate in the release of CV contents from reconstituted cortex; and (3) suggest that sulfhydryl-containing protein(s) present on the surface of purified CVs and plasma membrane are involved in exocytosis.

Key words: exocytosis, secretion, *N*-ethylmaleimide.

Introduction

Regulated exocytosis, the release of stored secretory products in response to a stimulus at the cell surface, is a characteristic feature of complex multicellular organisms. The set of stimuli to which a secretory cell can respond is determined by the specific array of surface receptors and ion channels that it expresses. Activation of most, if not all, of these receptors triggers an increase in the intracellular concentration of calcium ion, which stimulates exocytosis (Penner and Neher, 1988).

The sea urchin egg is a particularly convenient system for studying the regulated form of exocytosis. In the mature egg, a specialized set of secretory vesicles known as cortical vesicles (CVs) are firmly attached to the cytoplasmic face of the plasma membrane (PM). Upon fertilization, an increase in the cytosolic concentration of Ca²⁺ (Steinhardt *et al.* 1977; Turner *et al.* 1986) triggers the fusion of the CVs with the PM. This process deposits proteins and mucopolysaccharides from the CVs onto the surface of the egg, where they contribute to the formation of the fertilization envelope and the hyaline layer (Kay and Shapiro, 1985).

Fragments of egg cortex, consisting of the PM, the CVs and the vitelline layer, comprise an exocytotically competent system. When bathed in calcium-containing buffers, egg cortex undergoes a reaction at physiologically relevant concentrations of Ca²⁺ (Moy *et al.* 1983; Whitaker

and Baker, 1983) that results in the fusion of the CV and plasma membranes; and releases CV content proteins onto the extracytoplasmic surface of the PM (Whitaker and Baker; 1983; Chandler, 1984; Zimmerberg *et al.* 1985; Crabb and Jackson, 1985).

CV-free egg PM can be easily obtained by dislodging the CVs from cortical lawn (CL) preparations of egg cortex. This procedure produces a PM 'lawn', consisting of an array of PM fragments attached *via* their vitelline layer to a polylysine-coated microscope slide (Kopf *et al.* 1982; Crabb and Jackson, 1985). Fragments of egg cortex can be reconstituted by recombining purified CVs with a PM lawn (Crabb and Jackson, 1985; Whalley and Whitaker, 1988). The reconstituted cortical lawns (RLs) produced by this procedure appear to be exocytotically competent: Ca²⁺-containing buffers trigger a reaction that results in fusion of the CV and plasma membranes, and the deposition of CV contents onto the extracytoplasmic surface of the PM (Crabb and Jackson, 1985). While it remains to be determined whether reassociation correctly reconstitutes the CV-PM junction, the results of binding specificity and protease inhibition experiments suggest that reassociation may be a specific, protein-mediated event (Jackson and Modern, 1990).

The observation that cortical exocytosis can be inhibited by sulfhydryl-modifying agents such as *N*-ethylmaleimide (NEM; Haggerty and Jackson, 1983; Jackson *et al.* 1985) suggests that a sulfhydryl-containing protein may be a

part of the exocytotic apparatus of the cell. As an additional measure of the authenticity of reconstitution, we have investigated the NEM sensitivity of reconstituted cortex. Our results suggest that the release of CV contents from reconstituted cortex and native cortex are mechanistically equivalent in that both can be inhibited with NEM. In addition, analysis of the exocytotic capability of cortex reconstituted from NEM-treated components demonstrates that the NEM-sensitive component is present both in purified CVs and PM.

Materials and methods

Materials

Strongylocentrotus purpuratus were maintained at 9–12°C in a refrigerated aquarium containing Instant Ocean sea water from Aquarium Systems (Mentor, OH). Soybean trypsin inhibitor (SBTI), NEM, poly-L-lysine (molecular weight 2×10^6), Pipes, KCl, $MgCl_2$, EGTA, DL-dithiothreitol (DTT), goat serum and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (whole molecule) were purchased from Sigma Chemical Co. (St Louis, MO). NH_4Cl , NaH_2PO_4 and NaCl were from Fischer Scientific (Pittsburg, PA). The mouse IgA monoclonal antibody to hyalin was prepared by Dr Carol Vater in this laboratory (Vater and Jackson, 1990).

Preparation of reconstituted cortical lawns (RLs)

PM lawns and cortical lawns (CLs) were prepared in soybean trypsin inhibitor (SBTI)-containing buffers, as previously described (Crabb and Jackson, 1985). CVs were dislodged from purified egg cell surface complex (CSC) by gentle homogenization in TKE buffer (50 mM Tris-HCl, 600 mM KCl, 5 mM EGTA, pH 8.0) containing $10 \mu g ml^{-1}$ SBTI, and purified by two rounds of differential centrifugation (Crabb and Jackson, 1985). PM lawns and CVs were recombined to form RLs as follows (Crabb and Jackson, 1985): samples of a CV suspension ($A_{490} \approx 10$) were brought to pH 6.8 by the addition of 1.0 M Pipes, pH 6.1, and 150- μl samples were drawn (with a 2 cm \times 2 cm wick of filter paper) into microscope slide chambers containing PM lawns. CVs were allowed to bind to the PM lawns for a period of 15 min. The coverglass was removed from the chamber and unbound CVs were washed away by dipping the slide five times into each of two 100-ml beakers of PKME buffer (50 mM Pipes, 425 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, pH 6.8) containing $1 \mu g ml^{-1}$ SBTI. Additional buffer (PKME containing $10 \mu g ml^{-1}$ SBTI) was added to each sample, and a coverglass was placed on the chamber. CV binding was quantitated by analysis of the amount of light scattered by bound CVs when the lawn was observed with dark-field optics (Jackson and Modern, 1990).

Analysis of exocytosis.

Exocytosis in CLs and RLs was initiated by drawing PKME buffer containing 1 mM free Ca^{2+} into the microscope slide chamber, and the extent of reaction was quantitated by light-scattering analysis (Zimmerberg *et al.* 1985; Crabb and Jackson, 1986). Cortical exocytosis in CSC was quantitated by a turbidity-based assay (Sasaki and Epel, 1983; Haggerty and Jackson, 1983). Fusion of the CV and plasma membranes was confirmed by an immunofluorescence-based assay that detects the vectorial transfer of hyalin, a CV content protein, across the PM (Crabb and Jackson, 1985). Briefly, CLs from which most CVs had been removed by shearing with PKME buffer, or RLs, were prepared on coverglasses and placed in a coverglass rack. Exocytosis was initiated by submerging the rack into a beaker containing PKME buffer with 1 mM free Ca^{2+} . After a 20-s incubation, the reaction was stopped and the samples fixed by submerging the rack into a beaker containing PKME buffer with 1% glutaraldehyde, for 20 min. Glutaraldehyde was removed by washing once for 15 min with PK₄₇₅ME (same as PKME, but with 475 mM KCl), and twice for 15 min with PKME. Positive controls were permeabilized by incubating for 15 min in PKME containing 0.5% Triton X-100,

and washed twice for 10 min with PKME. Non-specific sites were blocked by incubation for 30 min with PKME/5% normal goat serum. Samples were incubated for 1 h with a 1/200 dilution of CV-69 ascites fluid (a hyalin-specific murine monoclonal antibody; Vater and Jackson, 1990) in PKME/5% normal goat serum, followed by three 10-min washes in PKME. Next, the samples were treated with a 1/250 dilution of FITC-conjugated goat anti-mouse IgG in PKME/5% normal goat serum, washed three times (10 min each) in PKME, mounted on chamber slides, and observed. Paired phase-contrast and immunofluorescence micrographs were taken of identical fields using Ilford HP5 film (ASA=400, push developed to ASA=800) and a Zeiss universal microscope equipped with a $\times 63$ Planapo objective. Measurements were made with a stage micrometer.

Reaction conditions for NEM modification

NEM-treated fractions were prepared as follows: CSC and CVs in TKE buffer containing $10 \mu g ml^{-1}$ SBTI and $1 \mu g ml^{-1}$ leupeptin were brought to 5 mM NEM and incubated for the indicated period of time (usually 15 min) at 20°C. NEM was added from a freshly prepared 200 mM stock solution in 0.5 M KCl. Reactions were terminated by the addition of DTT (from a 1.0 M stock) to a final concentration of 10 mM. CLs, RLs and PM lawns were incubated with 5 mM NEM in PKME buffer containing $10 \mu g ml^{-1}$ SBTI and $1 \mu g ml^{-1}$ leupeptin for the indicated period of time (usually 30 min) at room temperature. Reactions were terminated by dipping each slide five times into a 100 ml beaker containing PKME buffer with 1 mM DTT, and five times into a beaker containing PKME buffer without DTT. The washed samples were flooded with PKME buffer containing $10 \mu g ml^{-1}$ SBTI, $1 \mu g ml^{-1}$ leupeptin, and a coverglass was placed on each chamber.

Results

NEM inhibits exocytosis in CLs

We have previously shown that treatment of suspensions of egg cortex (CSC preparation) with NEM inhibits exocytosis by increasing the threshold Ca^{2+} concentration required to elicit reaction (Jackson *et al.* 1985). At pH 8.0, complete inactivation of CSC to a challenge by buffers containing 1 mM free Ca^{2+} required 15 min with 5 mM NEM (Fig. 1A). Similar results were obtained with fragments of egg cortex attached to poly-L-lysine-coated glass slides (CL preparations, Fig. 1B). CLs that had been treated at pH 6.8 with 5 mM NEM for the indicated period of time were challenged with a buffer containing 1 mM free Ca^{2+} and exocytosis was assessed by light-scattering analysis. Under these conditions, complete inactivation was achieved within 30 min. The control (Fig. 1B, open symbol) showed that a 30 min incubation in the absence of NEM is not inhibitory. Though it appears from the data presented in Fig. 1 that there is a difference in the rates of inactivation of the CSC (Fig. 1A) and CL (Fig. 1B) samples, this difference is due to the higher pH used in the CSC inactivation experiment (thiolate anions are more readily modified than thiols). At pH 6.8, the rate of inactivation of CSC is comparable to that of CLs (compare Fig. 1B with the NEM inactivation data of Jackson *et al.* 1985). The lag in the CSC and CL inhibition curves (Fig. 1A and 1B) reflects the fact that mild inactivation (low NEM concentration or short reaction time) increases the Ca^{2+} threshold, but does not prevent 100% release in response to a strong stimulus (1 mM Ca^{2+}). As previously noted (Jackson *et al.* 1985), this behavior suggests that a small fraction of the total number of NEM-sensitive proteins may be sufficient to support 100% exocytosis.

The use of higher than physiological concentrations of Ca^{2+} in these and subsequent experiments was necessitated by the characteristics of the CL and reconstituted

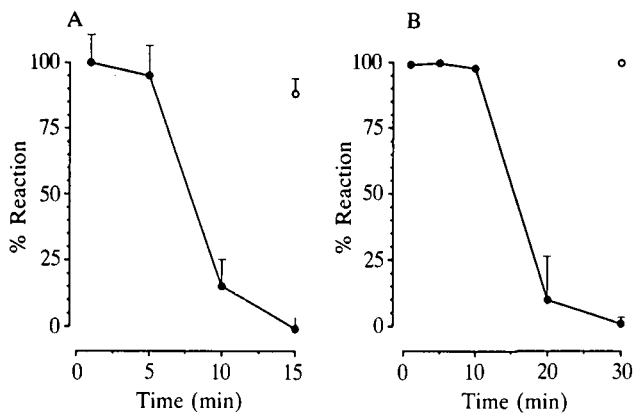


Fig. 1. NEM inhibits exocytosis in CSC and CLs. A. CSC (●—●) in TKME buffer, pH 8.0, was treated with 5 mM NEM at 20°C for the indicated times, as described in Materials and methods. Control CSC (○) was incubated in the same buffer without NEM. Exocytosis was initiated by diluting samples of the NEM-treated CSC into cuvettes containing a buffer with 1 mM free Ca^{2+} . Percentage reaction was determined by the turbidimetric procedure referred to in Materials and methods, with the turbidity change of the untreated control assigned a value of 100% reaction. Results are the mean \pm s.d. of triplicate samples, and are representative of three similar experiments. B. Cortical lawns (●—●) were treated with 5 mM NEM in PKME, pH 6.8, buffer for the indicated time at room temperature. Control lawns (○) were incubated in the same buffer without NEM. Exocytosis was initiated by drawing a buffer containing 1 mM free Ca^{2+} into the slide chamber. Percentage reaction was determined by light-scattering analysis. Each data point represents the mean \pm s.d. of triplicate samples. In this and subsequent figures, data points without error bars indicate that the s.d. was less than the size of the data point. Results are representative of three similar experiments.

lawn (RL) systems. CLs and RLs are half-maximally reactive at 5 and 36 μM Ca^{2+} , respectively; both are 100% reactive at 1 mM Ca^{2+} . In order to compare NEM inhibition of RLs and CLs, it was necessary to choose a Ca^{2+} concentration that stimulated the same high level of reaction in both preparations. In the experiments demonstrating that both RLs and CLs are inhibited by NEM treatment, the use of a Ca^{2+} concentration that is well above the physiologically relevant range presents no problem, since loss of response to a potent stimulus is a stronger criterion than loss of response to a minimal stimulus. In the experiments demonstrating the activity of RLs prepared from NEM-treated components, the possibility that reaction at 1 mM Ca^{2+} may occur *via* a different mechanism must be considered. While this possibility cannot be eliminated, the available evidence suggests that reactions at low and high Ca^{2+} may be equivalent: both occur *via* CV-PM fusion, and both are inhibitable by NEM treatment (see below).

NEM inhibits exocytosis in RLs

We were interested in the susceptibility of RLs to NEM inhibition because it provides an additional criterion by which the authenticity of reconstitution can be judged. If reconstitution correctly reassembles a functional CV-PM junction, it should be possible to inhibit RLs with NEM. To test this possibility, RLs and CLs were prepared as described in Materials and methods, incubated with or

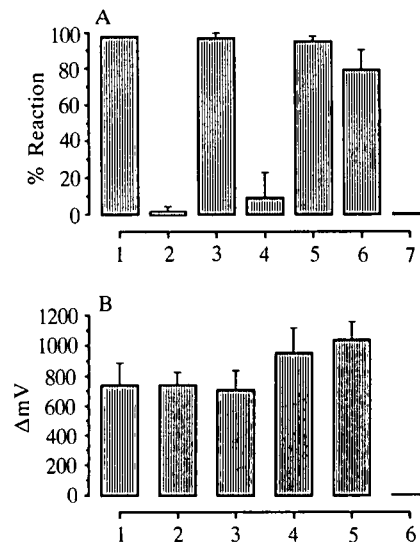


Fig. 2. Activity of RLs prepared from NEM-treated components: comparison with CLs, RLs and NEM-treated RLs. A. The following cortex preparations were tested for exocytotic capability as described in the legend to Fig. 1B: (1) untreated CLs; (2) CLs that had been NEM-treated for 30 min at room temperature; (3) untreated RLs; (4) RLs that had been NEM-treated for 30 min at room temperature; (5) RLs prepared from NEM-treated PM; (6) RLs prepared from NEM-treated CVs; (7) RLs prepared from NEM-treated CVs and NEM-treated PM. B. In addition to their reactivity, the RL samples (A, 3–7) were also analyzed for CV binding by the light-scattering technique described in Materials and methods. Column 1, Untreated RLs; 2, RLs that had been NEM-treated for 30 min at room temperature; 3, RLs prepared from NEM-treated PM; 4, RLs prepared from NEM-treated CVs; 5, RLs prepared from NEM-treated CVs and NEM-treated PM. The results presented in A, and in columns 1–5 of B are the mean \pm s.d. of six to eight determinations from three independent experiments. For comparison, column 6 of B shows that the Δ mV for unconstituted PM lawns in CV binding experiments is negligible (1.0 ± 2.6 mV; $n=13$ experiments). Under these same conditions the Δ mV for native cortical lawns is approximately 4000 mV (see Fig. 1 of Jackson and Modern, 1990).

without 5 mM NEM for 30 min, and challenged with PKME buffer containing 1 mM free Ca^{2+} . The extent of reaction was quantitated by light-scattering analysis, and is expressed in terms of percentage of reaction in order to facilitate comparison of CLs that contain many CVs with RLs that contain fewer. The results of this experiment (Fig. 2A, columns 1–4) demonstrate that both RLs and CLs are susceptible to inhibition by NEM, and lend support to the hypothesis that the mechanism of exocytosis in native and reconstituted cortex is the same.

Activity of RLs prepared from NEM-treated components

RLs are prepared by recombination of purified CVs with PM lawns. By pretreating each of these components with NEM prior to reconstitution it should be possible to determine whether the NEM-sensitive protein is located on the CVs, the PM, or both. To this end, the exocytotic capability of RLs that had been prepared with the four possible combinations of NEM-treated and untreated components was tested. In order to be sure that the NEM treatment was sufficient to inactivate the purified components, we chose conditions known to be sufficient to

inactivate cortex preparations. Thus, purified CVs were NEM-treated under conditions (5 mM NEM for 15 min at pH 8.0) that completely inactivate a suspension of cell surface complex (Fig. 1A), and PM lawns were NEM-treated under conditions (5 mM NEM for 30 min at pH 6.8) that completely inactivate CLs (Fig. 1B).

Surprisingly, RLs in which either the PM or the CVs had been NEM-treated retained activity to 1 mM Ca^{2+} (Fig. 2A, columns 5 and 6), whereas RLs in which both the CVs and PM had been NEM-treated were inactive (Fig. 2A, column 7). Controls showed that untreated CLs and RLs were active (Fig. 2A, columns 1 and 3), and that the NEM treatment was sufficient to inhibit CLs and RLs (Fig. 2A, columns 2 and 4). This result suggests that NEM-sensitive protein(s) are present in both purified CV and PM preparations.

Analysis of CV binding revealed that NEM treatment did not inhibit the binding of CVs to PM (Fig. 2B). In fact, in two of three experiments, NEM-treated CVs bound somewhat better than untreated CVs. Untreated CVs bound as well to NEM-treated PM lawns as to untreated PM lawns in all three experiments. Thus, differences in the exocytotic capability of RLs prepared from NEM-treated components was not attributable to deficient CV binding.

In a variation of the above experiment, we attempted to prepare RLs by recombination of untreated PM lawns with CVs that had been prepared from NEM-inactivated CSC. This variation produced CV suspensions that were significantly more dilute than those produced by the standard procedure. RLs formed from these dilute CV suspensions had a small extent of CV binding, and were not thoroughly examined. However, the few samples that were tested were observed to undergo exocytosis in response to 1 mM Ca^{2+} (data not shown).

In early work with this system (before routine determination of CV turbidity) we compared the binding of CVs prepared from equal concentrations of CSC and NEM-treated CSC, and observed that CVs prepared from the NEM-treated sample appeared to bind poorly to PM lawns (Crabb and Jackson, 1985). It is now clear that this result was due to the negative impact of NEM treatment on the yield of CVs. In these early experiments we also observed impaired CV binding to PM lawns that had been prepared from NEM-treated eggs. Reinvestigation of this observation revealed that prolonged treatment with 10 mM NEM (40 min at 20°C) does impair CV binding; though not uniformly. Many of the PM fragments in RLs prepared with these samples were virtually devoid of bound CVs, but some seemed to bind a near normal complement. Under milder conditions (10 mM NEM for 5 min at 20°C, in sea water buffered to pH 8) cortical exocytosis could be inhibited without any apparent inhibition of CV binding. These results suggest that the impaired binding of PM lawns prepared from NEM-treated eggs is not related to the ability of NEM to inhibit the cortical reaction.

The fact that untreated PM can rescue NEM-inactivated CVs and that untreated CVs can rescue NEM-treated PM provides evidence that inactivation by NEM is not simply due to steric hindrance of CV-PM contact (if it were, an untreated fraction should not be able to rescue a NEM-inactivated fraction). More important, the data show that both the CV membrane and the plasma membrane contain molecules that are capable of supporting exocytosis, in the reconstituted system. The fact that the sulphhydryl-containing protein(s) from either membrane alone are sufficient to support exocytosis suggests that sulphhydryl-containing

protein(s) from both membranes probably participate in the release of CV contents from untreated RLs and CLs.

Evidence for transfer of CV contents across the PM

Although untreated CVs and PM seemed to be capable of rescuing their NEM-inactivated counterparts, it was necessary to demonstrate that CV contents were released *via* exocytosis. The mechanism of release was investigated with an immunofluorescence-based assay that permits visualization of the transfer of CV content proteins across the PM (Crabb and Jackson, 1985). We have previously used this technique to demonstrate that Ca^{2+} stimulation of CLs and RLs results in the transfer of CV contents across the PM (Crabb and Jackson, 1985). When a CV in a CL or RL fuses with the PM, its contents are transferred across the PM into a dome-shaped compartment that is bounded on one side by the egg PM and on the other by the coverglass to which the lawn is attached. Within these compartments, secreted CV components (e.g. hyalin) are protected from exogenously added probes (e.g. antibodies). Immunofluorescence analysis of samples whose membranes have been disrupted by detergent is used to confirm the presence of CV contents within the domes (Crabb and Jackson, 1985).

Analysis of RLs prepared from NEM-treated components by this technique revealed that RLs in which either the CVs or PM had been NEM-treated reacted *via* exocytosis. Fig. 3 presents paired phase-contrast and fluorescence micrographs obtained with RLs prepared by recombination of NEM-treated PM lawns with untreated CVs. In the membrane-intact sample (Fig. 3A) hyalin contained in the domes in the center portion of the PM fragment (Fig. 3A, arrows in phase-contrast micrograph) was protected and did not combine with the anti-hyalin antibody. An imperfect seal between the PM and the coverglass allowed labelling of domes at the periphery of the PM fragment (Fig. 3A, arrows in fluorescence micrograph). Most free CVs (i.e. those bound to the coverglass rather than the PM) remained intact, and were not labelled with the anti-hyalin antibody. Detergent disruption of the membrane resulted in heavy labelling of all domes and free CVs (Fig. 3B), thereby confirming the presence of immunoreactive hyalin within these structures. Similar results were obtained with RL samples prepared by recombination of NEM-treated CVs with untreated PM lawns (Fig. 4): hyalin within intact domes was not labelled (Fig. 4A); hyalin within detergent-disrupted domes was heavily labelled (Fig. 4B).

These results are comparable to those obtained with the CL (Fig. 5) and untreated RL (Fig. 6) samples that served as positive controls for transfer of CV contents across the PM. The large domes formed from the densely packed CVs of CL samples (Fig. 5A, arrows in phase-contrast micrograph) present a different image than the small domes of RL samples (Fig. 6A, arrows); however, both protect entrapped hyalin from antibody labelling. The larger domes of CL samples are unstable and frequently rupture during processing. This can result in heavy labelling of domes in the interior as well as at the periphery of a fragment. The CL sample shown in Fig. 5A apparently contained three ruptured domes (arrows in fluorescence micrograph). Notice, however, that intact domes immediately adjacent to the those that ruptured remained unlabelled. In the detergent-disrupted sample (Fig. 5B) all domes were labelled.

These results provide strong evidence that RLs prepared

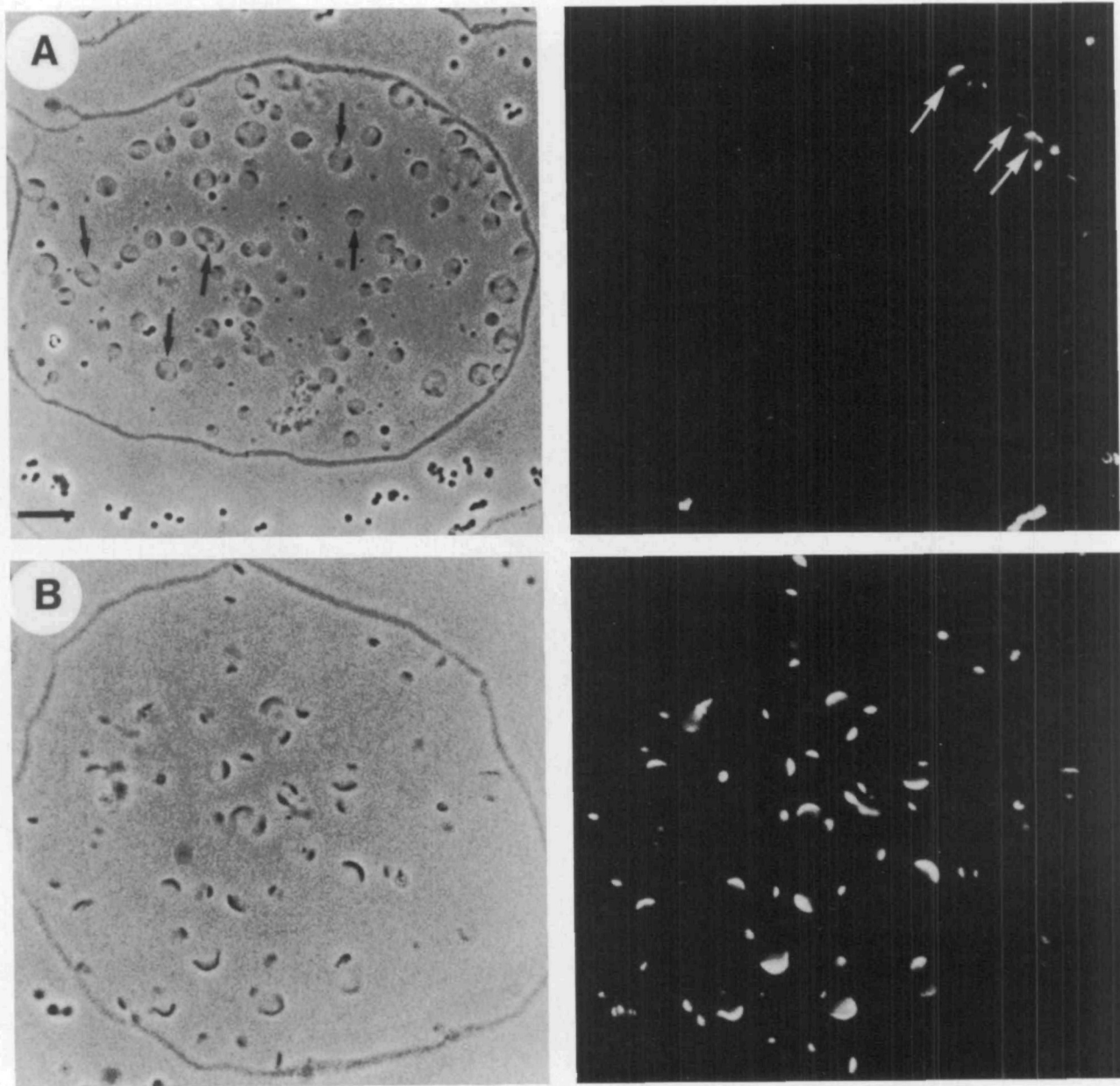


Fig. 3. CV content proteins are vectorially transferred across the PM in RLs prepared by recombination of NEM-treated PM with untreated CVs. Reconstituted lawns (RLs) were prepared from NEM-treated PM lawns and untreated CVs. Exocytosis was initiated by dipping the RLs into a buffer containing 1 mM free Ca^{2+} . At $t=15$ s, the reaction was terminated by dipping the samples into a buffer containing 1% glutaraldehyde. The fixed samples were probed for anti-hyalin immunofluorescence with an anti-hyalin monoclonal (1/250 dilution of the anti-hyalin ascites fluid). A. Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with intact membranes. Arrows in the phase-contrast image designate hyalin-containing domes that are not labelled with the anti-hyalin antibody. Labelled domes (denoted by arrows in the fluorescence image) are often seen at the circumference of the fragment. These apparently result from an imperfect seal between the coverglass and the membrane fragment. B. Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with detergent-disrupted membranes. Results presented in Figs 3–6 are representative samples from two independent experiments. Bar, 10 μm .

from NEM-treated components, like their untreated CL and RL counterparts, react *via* an exocytotic mechanism.

Discussion

The ability to prepare reconstituted egg cortex (RLs) by recombination of purified CVs with PM (Crabb and Jackson, 1985; Whalley and Whitaker, 1988) provides a potentially powerful tool for investigation of the molecular

mechanism of cortical exocytosis. To make full use of this technology it is important to demonstrate that the mechanism of reaction in RLs is equivalent to that of CLs, CSC and eggs. Several pieces of data support this hypothesis. (1) Binding specificity experiments suggest that reassociation of CVs with PM may be specific, protein-mediated event (Jackson and Modern, 1990). (2) Reassociation has been shown to be a prerequisite for the Ca^{2+} -triggered release reaction (Crabb and Jackson, 1985). (3) The Ca^{2+} -triggered release reaction results in the vectorial transfer

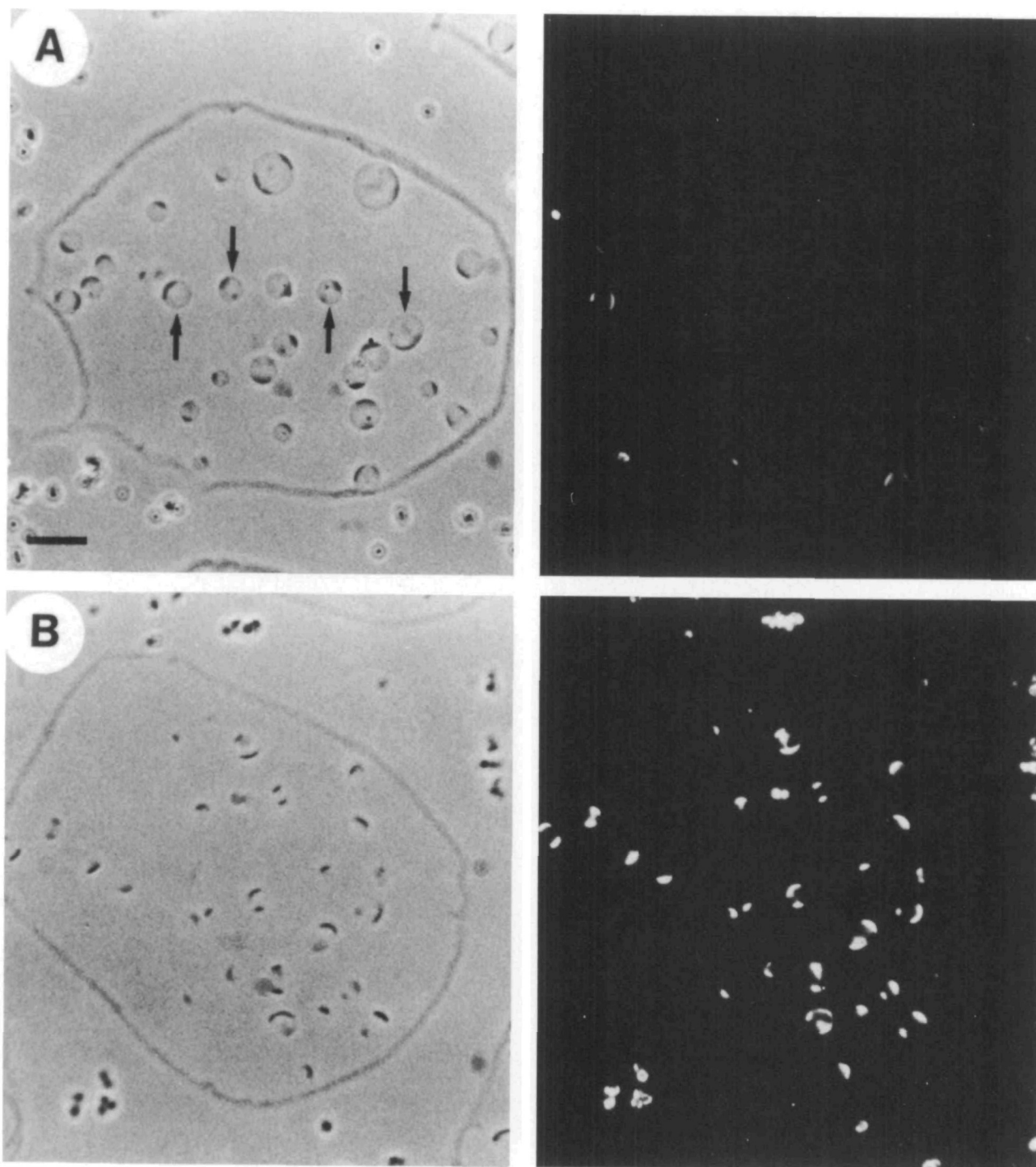


Fig. 4. CV content proteins are vectorially transferred across the PM in RLs prepared by recombination of NEM-treated CVs with untreated PM. Reconstituted lawns (RLs) were prepared from NEM-treated CVs and untreated PM lawns, as described in Materials and methods. Exocytotic transfer of CV contents across the PM was assessed by anti-hyalin immunofluorescence, as described in the legend to Fig. 3. **A.** Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with intact membranes. Arrows in the phase-contrast image designate hyalin-containing domes that are not labelled with the anti-hyalin antibody. **B.** Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with detergent-disrupted membranes. Bar, 10 μm .

of CV content proteins across the PM (Crabb and Jackson, 1985). (4) Despite the potentially detrimental dissociative procedures used to prepare RLs, their Ca^{2+} threshold (36 μM), though higher than that of CLs (5 μM), is low enough to be compatible with an exocytotic mechanism of release. To these we can now add the observation that RLs,

CLs, CSC and eggs are all susceptible to inhibition by NEM (Figs 1 and 2; and Jackson *et al.* 1985). Thus, in each case, at least one sulphhydryl-containing protein must be involved in the reaction. This finding is consistent with the hypothesis that the reactions are all mechanistically equivalent.

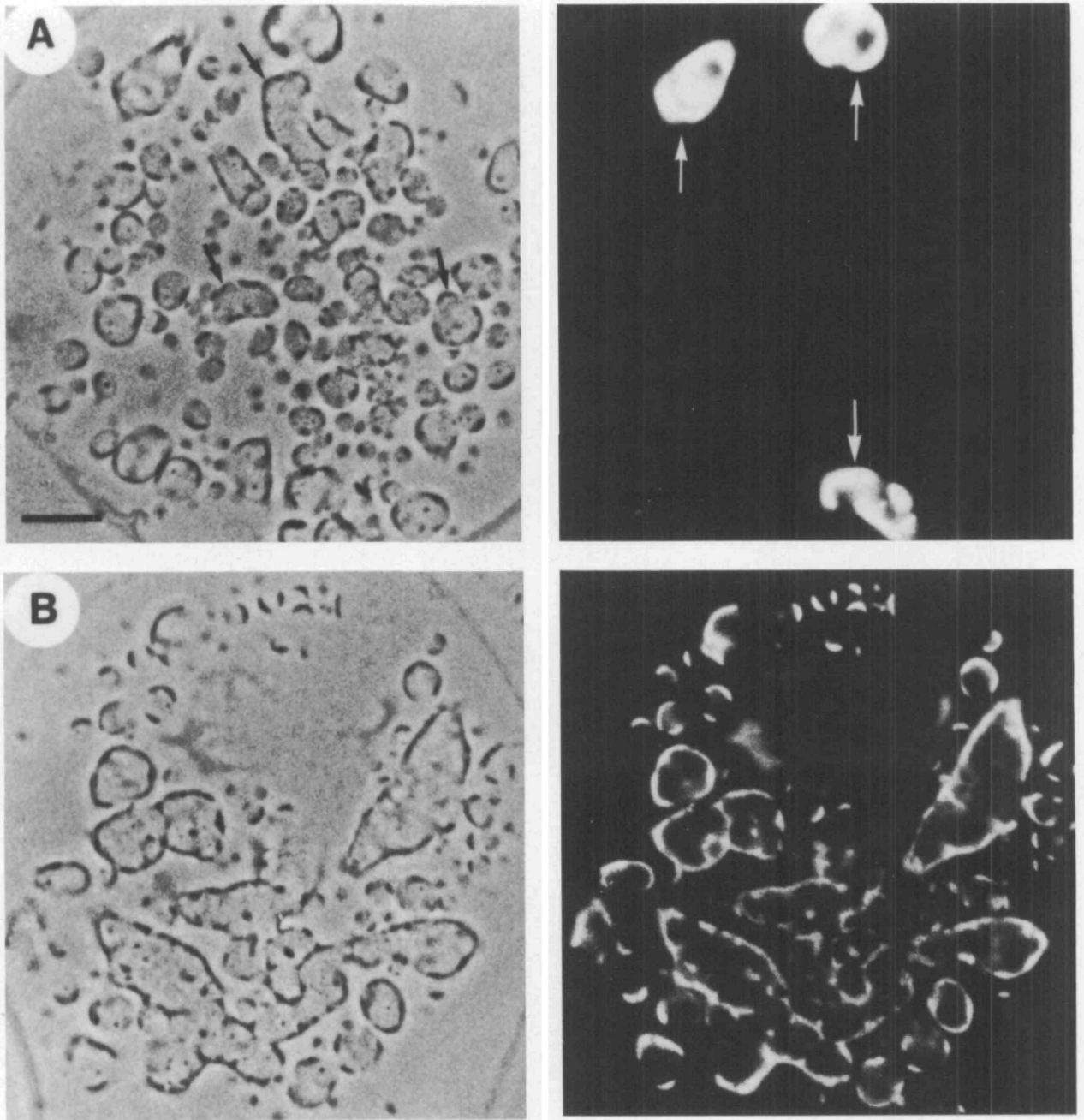


Fig. 5. CV content proteins are vectorially transferred across the PM in cortical lawns (CLs). Exocytotic transfer of CV contents across the PM of CLs was assessed by anti-hyalin immunofluorescence, as described in the legend to Fig. 3. A. Paired phase-contrast and immunofluorescent images of a cortical fragment with intact membranes. Arrows in the phase-contrast image designate large, hyalin-containing domes that are not labelled with the anti-hyalin antibody. Arrows in the fluorescence image designate ruptured domes that are heavily labelled with the anti-hyalin antibody. B. Paired phase-contrast and immunofluorescent images of a cortical fragment with detergent-disrupted membranes. Bar, 10 μm .

Investigation of the activity of RLs prepared from NEM-treated components demonstrated that untreated PM can rescue NEM-treated CVs and that untreated CVs can rescue NEM-treated PM. This surprising finding suggests that functionally equivalent NEM-sensitive protein(s) are present in both the CV and PM lawn preparations. At present it is not clear whether this shared activity is the result of a single protein located on both organelles, or of different yet functionally equivalent proteins. It is also not clear whether these protein(s) are located on both CV

membranes and plasma membranes in the intact egg; however, an interesting precedent for colocalization is provided by the *sec4* protein of yeast. *sec4* mutants are defective in constitutive secretion at a post-Golgi stage (Novick *et al.* 1980). The *sec4* protein has been found to be tightly associated with both PM and secretory vesicles (Goud *et al.* 1988). Mutational analysis suggests that it regulates vesicular traffic by cycling between the PM and secretory vesicles (Walworth *et al.* 1989); thus, *sec4* is located on both secretory vesicles and PM and is required

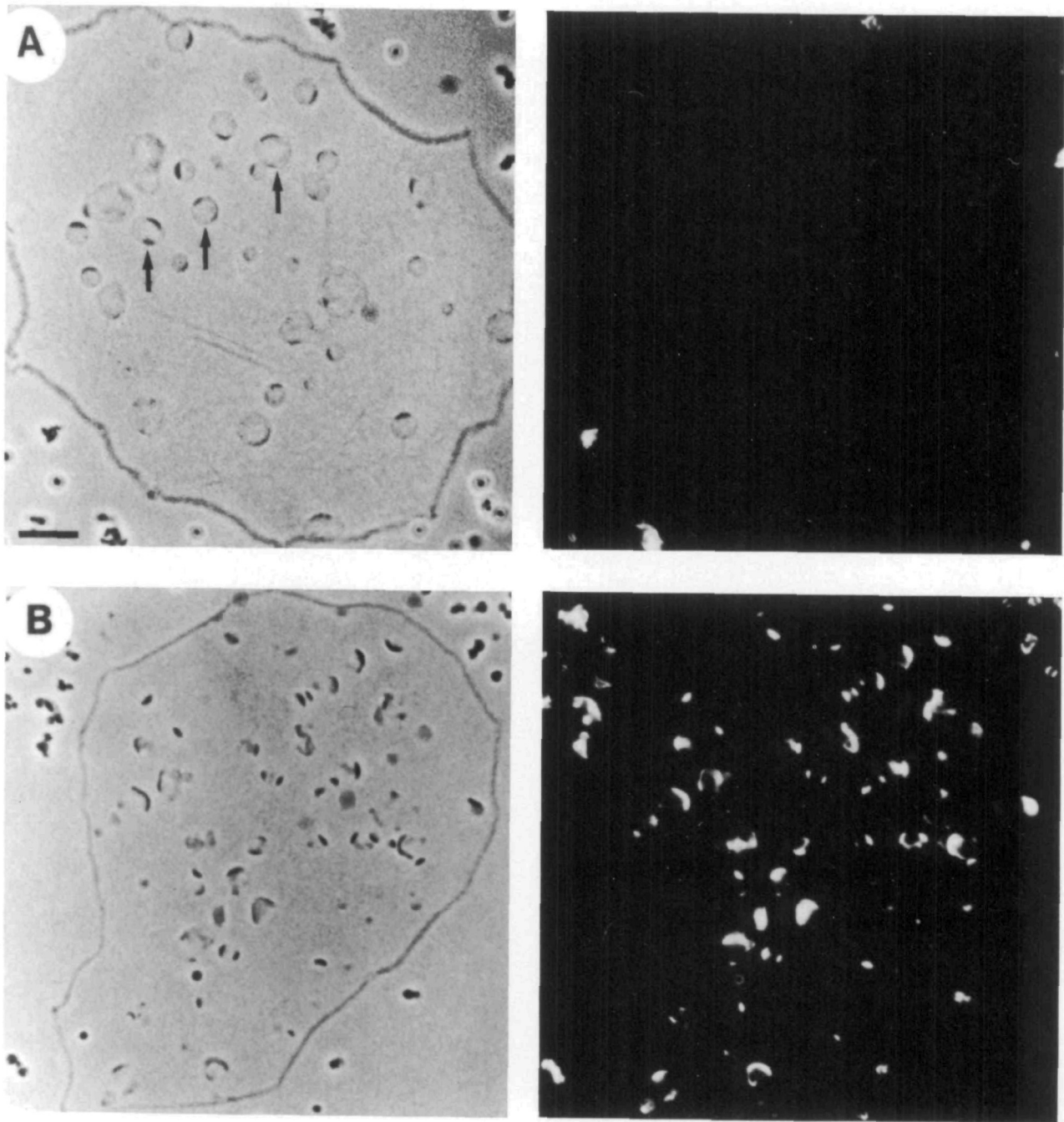


Fig. 6. CV content proteins are vectorially transferred across the PM in RLs prepared by recombination of untreated CVs and PM. Exocytotic transfer of CV contents across the PM of an RL prepared by recombination of untreated CVs with PM lawns was assessed by anti-hyalin immunofluorescence, as described in the legend to Fig. 3. A. Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with intact membranes. Arrows in the phase-contrast image designate hyalin-containing domes that are not labelled with the anti-hyalin antibody. B. Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with detergent-disrupted membranes. Bar, 10 μ m.

for constitutive exocytosis. Another possibility is that the NEM-sensitive protein(s) may reside at the CV-PM junction. Dislodgement of CVs from the PM could then result in the distribution of the protein(s) to both membranes. Alternatively, in the intact egg, the protein(s) could be exclusively located on one membrane and become redistributed to the other during fractionation.

The rescue experiments also suggest that NEM inhibition does not result from steric inhibition of CV-PM

contact. Steric inhibition should be dominant, i.e. the steric constraints preventing membrane contact should not be removed by supplying an unmodified partner. The fact that unmodified fractions can restore function implies that the unmodified fraction is capable of actively promoting exocytosis, i.e. it supplies an essential function. On the other hand, RLs prepared from NEM-treated components could react *via* a qualitatively different mechanism than CLs and untreated RLs, but the results of the vectorial

transfer experiments (Figs 3–6) suggest that this is not the case. Rather, it seems that both CVs and the PM carry proteins capable of promoting exocytosis. This is consistent with the observation that large aggregates of CVs can fuse with each other, even in the absence of exogenously added PM (Crabb and Jackson, 1985). It is also consistent with the phenomenon of compound exocytosis, in which secretory vesicles fuse with each other, as well as with the PM, in cells that undergo massive and concerted exocytotic reactions, e.g. mast cells (Rohlich *et al.* 1971), parotid acinar cells (Amsterdam *et al.* 1969) and eggs (Chandler, 1984).

The precise role of NEM-sensitive protein(s) in cortical exocytosis cannot be determined from the data that are currently available. The simplest hypothesis that accommodates the data suggests that the NEM-sensitive protein(s) may provide an essential function; however, the observation that mild proteolysis can reverse NEM inhibition of CSC (Jackson *et al.* 1985), CL and RL samples (Jackson and Modern, unpublished results) indicates that the sensitive sulfhydryl group is not at the active site. It is likely that it is located on a regulatory domain that is non-functional (i.e. inhibitory) when modified. The putative regulatory domain could be either covalently or non-covalently associated with the domain that is essential for exocytosis. Thus the NEM-sensitive protein(s) must either supply an essential function or be so closely associated with an essential protein that modification of the NEM-sensitive protein(s) interferes with the activity of the essential protein. It is tempting to speculate that the NEM-sensitive protein(s) of egg cortex may be related to the 78 000 M_r NEM-sensitive factor (NSF) identified by Rothman and his colleagues (Block *et al.* 1988; Wilson *et al.* 1989). NSF has been shown to be required for membrane fusion steps at several stages of the secretory (Glick and Rothman, 1987; Beckers *et al.* 1989) and endocytic pathways (Diaz *et al.* 1989), but its precise role in these events is also unknown. In any case, identification and characterization of the NEM-sensitive protein(s) of egg cortex is clearly essential for the understanding of the molecular mechanism of regulated exocytosis in the egg, and perhaps in other cell types as well.

We thank Dr Carol Vater for generously providing anti-hyalin antibody. This research was supported by grant GM 26763 from the National Institutes of Health.

References

- AMSTERDAM, A., OHAD, I. AND SCHRAMM, M. (1969). Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle. *J. Cell Biol.* **41**, 753–773.
- BECKERS, C. J. M., BLOCK, M. R., GLICK, B. S., ROTHMAN, J. E. AND BALCH, W. E. (1989). Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature* **339**, 397–398.
- BLOCK, M. R., GLICK, B. S., WILCOX, C. A., WIELAND, F. T. AND ROTHMAN, J. E. (1988). Purification of an *N*-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7852–7856.
- CHANDLER, D. E. (1984). Exocytosis *in vitro*: Ultrastructure of the isolated sea urchin cortex as seen in platinum replicas. *J. Ultrastruct. Res.* **89**, 198–211.
- CRABB, J. H. AND JACKSON, R. C. (1985). *In vitro* reconstitution of exocytosis from plasma membrane and isolated secretory vesicles. *J. Cell Biol.* **101**, 2263–2273.
- CRABB, J. H. AND JACKSON, R. C. (1986). Polycation inhibition of exocytosis from sea urchin egg cortex. *J. Membr. Biol.* **91**, 85–96.
- DIÁZ, R., MAYORGA, L. S., WEIDMAN, P. J., ROTHMAN, J. E. AND STAHL, P. D. (1989). Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature* **339**, 398–400.
- GLICK, B. S. AND ROTHMAN, J. E. (1987). Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature* **326**, 309–312.
- GOUD, B., SALMINEN, A., WALWORTH, N. C. AND NOVICK, P. J. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane of yeast. *Cell* **53**, 753–768.
- HAGGERTY, J. G. AND JACKSON, R. C. (1983). Release of granule contents from sea urchin egg cortices: new assay procedures and inhibition by sulfhydryl-modifying reagents. *J. biol. Chem.* **258**, 1819–1825.
- JACKSON, R. C., HAGGERTY, J. G. AND WARD, K. K. (1985). Mild proteolytic digestion restores exocytotic activity to *N*-ethylmaleimide-inactivated cell surface complex from sea urchin eggs. *J. Cell Biol.* **101**, 6–11 (Erratum, **101**, 1167).
- JACKSON, R. C. AND MODERN, P. A. (1990). Reassociation of cortical secretory vesicles with sea urchin egg plasma membrane: assessment of binding specificity. *J. Membr. Biol.* (in press).
- KAY, E. AND SHAPIRO, B. M. (1985). The formation of the fertilization membrane of the sea urchin egg. In *Biology of Fertilization* (ed. C. B. Metz and A. Monroy), vol. 3, pp. 45–80. Academic Press, Orlando, FL.
- KOPF, G. S., MOY, G. W. AND VACQUIER, V. D. (1982). Isolation and characterization of sea urchin egg cortical granules. *J. Cell Biol.* **95**, 924–932.
- MOY, G. W., KOPF, G. S., GASCHÉ, C. AND VACQUIER, V. D. (1983). Calcium-mediated release of glucanase activity from cortical granules of sea urchin eggs. *Dev. Biol.* **100**, 267–274.
- NOVICK, P., FIELD, C. AND SCHERMAN, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**, 205–215.
- PENNER, R. AND NEHER, E. (1988). The role of calcium in stimulus-secretion coupling in excitable and non-excitable cells. *J. exp. Biol.* **139**, 329–345.
- ROHLICH, P., ANDERSON, P. AND UVNAS, B. (1971). Electron microscopic observations on compound 48/80-induced degranulation in rat mast cells. *J. Cell Biol.* **51**, 465–483.
- SASAKI, H. AND EPEL, D. (1983). Cortical vesicle exocytosis in isolated cortices of sea urchin eggs: description of a turbidometric assay and its utilization in studying effects of different media on discharge. *Dev. Biol.* **98**, 327–337.
- STEINHARDT, R., ZUCKER, R. AND SCHATTEN, G. (1977). Intracellular calcium release at fertilization in the sea urchin egg. *Dev. Biol.* **58**, 185–196.
- TURNER, P. R., JAFFE, L. A. AND FEIN, A. (1986). Regulation of cortical vesicle exocytosis in sea urchin eggs by inositol 1,4,5-trisphosphate and GTP-binding protein. *J. Cell Biol.* **102**, 70–76.
- VATER, C. A. AND JACKSON, R. C. (1990). Immunolocalization of hyalin in sea urchin eggs and embryos using an antihyalin-specific monoclonal antibody. *Molec. Reprod. Dev.* (in press).
- WALWORTH, N. C., GOUD, B., KABCENELL, K. AND NOVICK, P. J. (1989). Mutational analysis of *SEC4* suggests a cyclical mechanism for the regulation of vesicular traffic. *EMBO J.* **8**, 1685–1693.
- WHALLEY, T. AND WHITAKER, M. (1988). Exocytosis reconstituted from the sea urchin egg is unaffected by calcium pretreatment of granules and plasma membrane. *Biosci. Rep.* **8**, 335–343.
- WHITAKER, M. AND BAKER, P. F. (1983). Calcium-dependent exocytosis in an *in vitro* secretory granule plasma membrane preparation from sea urchin eggs and effects of some inhibitors of cytoskeletal function. *Proc. R. Soc. Lond. B* **218**, 397–413.
- WILSON, D. W., WILCOX, C. A., FLYNN, G. C., CHEN, E., KUANG, W.-J., HENZEL, W. J., BLOCK, M. R., ULLRICH, A. AND ROTHMAN, J. E. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* **339**, 355–359.
- ZIMMERBERG, J., SARDET, C. AND EPEL, D. (1985). Exocytosis of sea urchin cortical vesicles *in vitro* is retarded by hyperosmotic sucrose: kinetics of fusion monitored by quantitative light scattering microscopy. *J. Cell Biol.* **101**, 2398–2410.

(Received 20 November 1989 – Accepted 2 March 1990)

